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TITLE: Expression of the proliferation antigen BM28 /hMCM2 in human tumors and normal tissue.
AUTHOR: Werness B A (Reprint); Todorov I T; Brooks J S J; Wang H Q; Buddharadju A; Todorova P D; Slocum H; Huberman J A
CORPORATE SOURCE: ROSWELL PK CANC INST, DEPT PATHOL, BUFFALO, NY 14263; ROSWELL PK CANC INST, DEPT MOL CELL BIOL, BUFFALO, NY 14263
COUNTRY OF AUTHOR: USA
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AUTHOR(S): Werness, Bruce A. Vvan T. Todorov (1); Brooks, John S. J.; Wang, Hui-Qun; Buddharadju, A.; Todorova, Pechka D.; Slocum, Harry; Huberman, Joel A.
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TITLE: Clone 10d/BM28 (CDCL1), an early S-phase protein, is an important growth regulator of melanoma.
AUTHOR: Spanjaard R A; Lee P J; Sarkar S; Goedegebuure P S; Eberlein T J
CORPORATE SOURCE: Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA.
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TITLE: HsMCM2/BM28: a novel proliferation marker for human tumors and normal tissues.
AUTHOR: Todorov I T; Werness B A; Wang H Q; Buddharaju L N; Todorova P D; Slocum H K; Brooks J S; Huberman J A
CORPORATE SOURCE: Department of Molecular and Cellular Biology, Roswell Park

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Clone 10d/BM28 (*CDCL1*), an Early S-Phase Protein, Is an Important Growth Regulator of Melanoma[1]

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ABSTRACT

Retinoic acid (RA) induces growth arrest and differentiation of many different tumor cells. RA activates RA receptors, which function as ligand-dependent transcriptional modulators. S91 murine melanoma cells stop proliferating and then reversibly differentiate into a melanocytic cell type after the administration of RA. The genetic changes that take place during this process serve as an excellent model for the etiology of melanoma. The use of subtractive hybridization techniques yielded several differentially expressed cDNAs that are associated with RA-induced growth arrest. One clone, cyclin D1, is repressed and is probably a differentiation marker. Two other cDNAs represent novel, RA-inducible genes. Expression of another cDNA, clone 10d, is strongly down-regulated. It is the homologue of the human gene BM28 (*CDCL1*) that is indispensable for entry into S phase and cell division. S91 cells that are permanently transfected with a plasmid that constitutively expresses clone 10d become significantly more resistant to RA, suggesting that repression of this gene is a critical event in RA-induced growth arrest. The use of reverse transcription-PCR for the detection of expression in human melanoma *in vitro* was performed to study the potential role of clone 10d/BM28 in this disease. It is expressed in 80% of melanoma cell lines but is virtually undetectable in primary melanocytes. The expression of BM28 is not regulated by RA in human, RA-resistant melanoma cells. These results suggest that clone 10d/BM28 functions as an important tumor cell growth promoter. The regulation of clone 10d may be directly mediated by RA receptors, and escape from negative regulation may, thus, contribute to the etiology of melanoma.

INTRODUCTION

RA[3] and other (synthetic) retinoids are known to cause growth arrest and differentiation of a large variety of tumor cells *in vitro* (1), and retinoids have also been used with some clinical success in the treatment and chemoprevention of different (premalignant) tumors (2–7).

The first step in this genetic cascade is relatively well established. The effects of RA are mediated by activation of specific nuclear hormone receptors, RARs, for which there are three separate genes (α, β, and γ). RARs are normally heterodimerized with the related RXRs, and this complex can bind to RA response elements in target genes and, subsequently, regulate their levels of expression (8–10). Unfortunately, the elucidation of the following downstream signaling pathway has proven to be very difficult, and at present, little is known about the identity of the RAR target genes and how they participate in the genetic program of these cells. Identification of these genes may shed new light on why certain cell lines and cancers are responsive to RA and others are not.

S91 murine malignant melanoma cells serve as a useful genetic model for the development of melanoma because they become growth arrested after the addition of RA (within 24 h) and then differentiate into a benign, morphologically distinct melanocytic cells (after about 5 days of treatment; Refs. 11–14). We recently showed that RA-dependent growth arrest of S91 cells, which express RXRα, RXRβ, RARα, RARγ, and low levels of RARβ (12–14), can be mediated by all three RAR isoforms and also by RXRα and/or RXRβ but that morphological differentiation is specifically mediated by RARγ (15). Here, we have focused our efforts on the cloning and functional characterization of RA-dependent target genes that are specifically associated with induction of growth arrest. Expression of these genes may be aberrant in neoplastic disease and contribute to its uncontrolled growth, and thus, their identification in the S91 model may provide new insight into the etiology of melanoma and, perhaps, other cancers as well.

Techniques like subtractive hybridization (16) and "differential display" (17) have been used successfully in the past for cloning of melanoma- and melanocyte-specific genes from several different cell lines (18–24). Unfortunately, the analyses of these often partial cDNAs usually do not go beyond mRNA expression studies. Although this has provided interesting correlative information, the physiological relevance of many of these gene fragments remains to be established.

In our experimental protocol, we treated S91 cells with RA until they were completely growth arrested but, morphologically, still unchanged. We then applied subtractive hybridization methods to clone RA-regulated genes that are associated with this process. We obtained several differentially expressed cDNAs (up- and down-regulated by RA), and analyzed their tissue distributions, kinetics of expression, and mechanisms of RA-dependent regulation. One cDNA that we obtained, clone 10d, is the murine homologue of the human gene BM28 (or *CDCL1*). This gene has previously been shown to be essential for DNA synthesis and cell division (25). Functional studies show that the repression of this gene largely mediates the induction of RA-dependent growth arrest. To investigate the putative role of this gene in human disease, we analyzed its expression in RA-resistant human melanoma cell lines by RT-PCR. We found that it is readily detectable in the majority of cell lines but that its expression level is extremely low in primary melanocytes. These results suggest that clone 10d/BM28 functions as a critical tumor cell growth promoter of melanoma cells. The expression of clone 10d may be directly repressed by RARs, and escape from negative regulation may, thus, contribute to the etiology of melanoma.

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assay. S91, B16, and A375 cells were obtained from the American Type Culture Collection. Human primary melanocytes were obtained from Clonetics (San Diego, CA) and grown with the manufacturer's medium. All "C"-numbered human melanoma lines were established in our laboratory from patient material. Tissue culture plates and flasks were obtained from Costar (Cambridge, MA). S91 cells were grown in DMEM (mouse lines) or RPMI 1640 (human lines) with 10% (v/v) FCS at 37°C in 5% CO_2 in humidified air. RA stock solutions (1 mM in DMSO) were made fresh and added to medium to achieve the desired concentration. Final

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[3] The abbreviations used are: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; RT, reverse transcription; CHX, cycloheximide; pfu, plaque-forming units.

DMSO concentrations in cell culture medium never exceeded 0.1% (v/v). For the cell proliferation assay, cells were seeded at a density of about 7500 cells/well in triplicate in 96-well plates and treated for 5 days in total with RA or DMSO. Medium was changed every 24 h. On day 5, relative viable cell numbers were obtained using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). A_{490} nM was determined in a UV Max Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

Establishment of Permanently Transfected Cell Lines. An *Xho*I-*Xba*I fragment containing the coding region of clone 10d was subcloned into pcDNA3, yielding plasmid pcDNA10d. pcDNA3 constitutively expresses cloned cDNAs *via* the cytomegalovirus promoter and also has a gene conferring resistance to neomycin (Invitrogen, San Diego, CA). Plasmids were grown in *Escherichia coli* strain DH5α in Luria-Bertani medium with ampicillin and purified using a Qiagen (Chatsworth, CA) Plasmid Maxi Kit. For the transfections, cells were grown in large flasks (165 cm^2) until they were 75% confluent. Cells (1×10^7) were harvested by trypsinization, washed in ice-cold DMEM, resuspended in 0.5 ml of ice-cold DMEM in a 0.4-cm electroporation cuvette (Invitrogen), and incubated for 5 min on ice with 30 μg of pcDNA3 (control) or pcDNA10d. Cells were then electroporated using a Electroporator II (Invitrogen) set at 200 V and 1000 μF, incubated on ice for 10 min, resuspended in DMEM/10% FCS at 37°C, and plated on 150-cm^2 plates. After 48 h, 800 μg/ml G418 (Geneticin; Life Technologies, Inc., Grand Island, NY) was added to the medium to select for neo^r clones. Nineteen pcDNA10d and 5 pcDNA3 transfected cell lines were obtained by placing cloning cylinders (Bellco Glass, Vineland, NJ) over the colonies, after which they were harvested and individually propagated. pcDNA10d lines were analyzed by Southern blot for integration of the 10d cDNA, and two positive lines, pcDNA10d lines 5 and 15, were selected for further analysis (data not shown). In addition, control pcDNA3 transfectant cell lines 1 and 3 were used.

RNA Isolation and Northern Blot Analysis. Cells were grown in large plates (150 cm^2) until they were 75% confluent. Medium containing 1 μM RA or control (DMSO) and/or CHX, as indicated (Sigma Chemical Co., St. Louis, MO), was changed every day. Cells were harvested at the indicated time points and washed twice with PBS. Single-selected poly(A)$^+$ mRNA was extracted using a Qiagen Direct mRNA Midi Kit, double-selected poly(A)$^+$ mRNA was extracted using a Stratagene (La Jolla, CA) mRNA isolation kit, or total RNA was isolated using Trizol Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Two μg of mRNA or 15 μg total RNA were subjected to electrophoresis through a denaturing formaldehyde-agarose gel (1%). Blotting procedures, hybridization/washing conditions, and preparation of radioactively labeled probe were all performed as described (14). Cyclophilin probe serves as internal control for loading and blotting. Fold regulation of individual probes was determined by quantification by use of a PhosphorImager (Molecular Dynamics) with ImageQuant software.

Library Construction, Subtractive Hybridization, and RT-PCR. S91 cells (5×10^6) were treated with DMSO (control) or with 1 μM RA for 48 h. Double-selected poly(A)$^+$ mRNA was extracted (Stratagene), and 5 μg were used for the construction of two libraries in λZAPII, respectively, according to the manufacturer's protocol (Stratagene). Titers of the primary libraries were 6.2×10^6 pfu/μg for control and 5.8×10^6 pfu/μg for the RA-treated cells. After one round of amplification, titers were 8.6×10^9 and 7.8×10^9 pfu/ml, respectively.

Subtractive hybridization was essentially performed according to the method of Swaroop *et al.* (16). In short, a 500-fold excess of "driver RNA" over "tester single-stranded DNA" was used. The control or 0-h library was subtracted from the 48-h library, giving melanocyte-associated cDNAs, or "u"-series clones for "up-regulated by RA," and the 48-h RA-treated library was subtracted from the 0-h library, giving melanoma-associated clones, or "d"-series clones for "down-regulated by RA." Twenty-seven different clones were obtained, and their individual regulation by RA was tested by Northern blot analysis. Standard recombinant DNA procedures were followed. Dideoxy DNA sequencing of (partial) cDNAs was performed at the Howard Hughes Biopolymers Facility (directed by Dr. J. Rush), Harvard Medical School, Boston, MA. 5′-rapid amplification of cDNA ends was performed according to the manufacturer's protocol (Marathon Kit; Clontech, Palo Alto, CA), either directly from the library or *via* RT of mRNA to obtain full-length coding sequences for clones 7u and 10d.

RT reactions were performed according to the manufacturer's protocol (Superscript Preamplification System; Life Technologies, Inc.). Standard PCRs were performed with an M. J. Research, Inc. (Watertown, MA), PTC-100 Thermal Cycler, under the following conditions: immediately following preheating mix for 3′ at 94°C, 40 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C were performed. Primers were designed that matched common sequences in BM28 and clone 10d, respectively (generating equally sized fragments), and species-specific primers for human and mouse β-actin were used (generating differently sized cDNA fragments). Primer sequences will be given upon request.

***In Vitro* Transcription/Translation, Protein Gels, and Western Blots.** cDNAs were transcribed/translated *in vitro* in the presence of [^{35}S]methionine in the TNT-T7-Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's protocol. Proteins were separated by 7.5% SDS-PAGE, and gels were treated with Amplify (Amersham, Arlington Heights, IL) according to the manufacturer's instructions before drying and exposure by autoradiography. Western blots were performed as described previously (14). In short, 3×10^5 cells were harvested, lysed in Laemmli sample buffer, and separated by 8% SDS-PAGE. After transfer onto nitrocellulose filters, filters were blocked with 5% Carnation nonfat dry milk and probed with affinity-purified, polyclonal rabbit antihuman BM28 antibodies, followed by a second incubation with horseradish peroxidase-coupled goat antirabbit antibody and visualization of bands using the ECL chemoluminescence kit (Amersham).

RESULTS

Detection, Tissue Distribution, and Cloning of Differentially Expressed cDNAs. S91 melanoma cells were treated for 48 h with 1 μM RA or DMSO (control). RA treatment results in cells that are completely growth arrested but remain unchanged morphologically. We reasoned that, at this stage, we may expect to find relatively few changes in gene expression, but those genes that are regulated in this time frame may have a relatively high potential of being of functional importance for growth- and cell cycle-related processes.

We then applied a molecular screen using subtractive hybridization techniques to clone cDNAs that are differentially expressed during this period of RA treatment. Our search encompassed genes that are predominantly expressed in the fast proliferating melanoma phase of the cells (d-series), as well as those expressed in the growth-arrested cells (u-series). Theoretically, this strategy permits the discovery of of both potential tumor promoters and tumor suppressor genes, respectively. Twenty-seven different clones were obtained and analyzed for RA-dependent regulation by Northern blot. The blots were also probed for RARβ expression, a well-established inducible RAR target gene in these cells (12–14), and cyclophilin was included as an internal control. Ultimately, four cDNAs proved to be derived from differentially expressed genes that were either down- or up-regulated. The results for these cDNAs, named clones 5d, 10d, 7u, and 13u, are shown in Fig. 1.

The expression of clone 5d is readily detectable in the fast-growing cells, but it is strongly repressed after RA treatment (3.1-fold). Two mRNA species are seen. The longer, much more abundant transcript is 4 kb long, and the shorter one is about 3.5 kb long. This clone is identical to cyclin D1 (*Cyl*-1; Refs. 26 and 27). Both transcripts are derived from the same gene; the shorter form is thought to arise through termination at an internal poly(A) tract (28). Overexpression of cyclin D1 has been implicated in the etiology of many types of cancer, particularly in parathyroid and breast cancer (29–31). B16 melanoma cells, which are very similar to S91 cells, can be induced to undergo terminal differentiation with L-tyrosine, which is also accompanied by repression of cyclin D1 expression (however, CDK4 levels remain the same; Ref. 32). Thus, in this cell system, cyclin D1 likely represents a marker for melanocytic differentiation and irreversible cell cycle arrest rather than an RAR target gene that can be reversibly regulated.

The clone 10d probe hybridizes with a mRNA that is about 3.8 kb long and is only detectable in the proliferating melanoma cells (re-

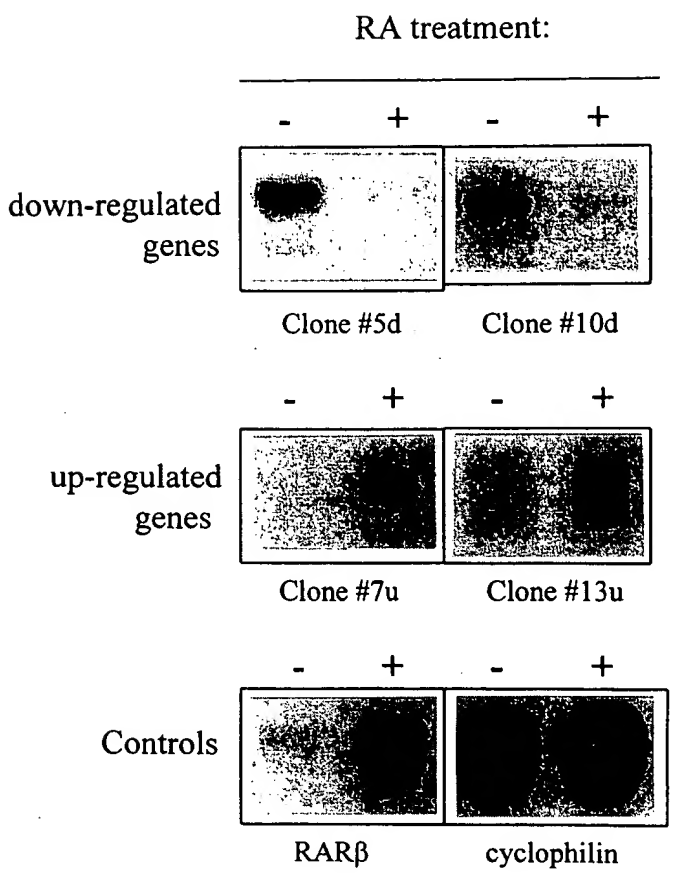

Fig. 1. Northern blot analysis (1.5 μg of double-selected poly(A)$^+$ RNA/lane) of differentially expressed (down- and up-regulated) genes in S91 cells that are associated with growth arrest, after 2 days of treatment with 1 μM RA (+) as compared to control (DMSO-treated) cells (−). Induction of expression of RARβ serves as positive control, whereas cyclophilin expression remains unchanged. mRNA sizes are estimated to be 4.0 and 3.5 kb for clone 5d, 3.8 kb for clone 10d, 1.4 kb for clone 7u, and 1.35 and 1.4 kb for clone 13u.

pressed by 5-fold). In contrast, clone 7u is virtually undetectable in those cells, but it is induced in the growth-arrested cells by 7.1-fold. It detects a mRNA of about 1.4 kb. Finally, clone 13u can be detected in control as well as RA-treated cells, but its expression is induced 2.3-fold in the latter. Interestingly, the probe detects two mRNA species that are about 1.35 and 1.4 kb long and may represent alternative splice variants. By comparison, the "classic" positive control, RARβ, is induced 17.4-fold.

Next, we characterized the *in vivo* expression patterns of clones 10d, 7u, and 13u. Total RNA was isolated from various normal mouse tissue, and expression was determined by Northern blot analysis (Fig. 2). The clones show a surprisingly diverse and selective expression pattern. Clone 10d is mostly expressed in the spleen and, at very low levels, in testis and brain. Clone 7u is mainly expressed in the large intestine and, at low levels, in lung and brain. Finally, expression of clone 13u is more widely distributed, including in the brain (in particular, the cerebrum), but it is expressed most notably in testis (this gel did not resolve the two transcripts seen in Fig. 1). Interestingly, expression in melanoma and testes has been reported before for three human gene families named *MAGE*, *BAGE*, and *GAGE*, which generate MHC class I-restricted tumor antigens in human melanoma (33–35). The restrictive nature of expression of these clones indicates that they may have a specialized function in these tissues.

Sequence Analysis of Clones 7u, 13u, and 10d: Clone 10d Is the Homologue of BM28. Sequence analysis of clones 7u and 13u reveals no obvious homologies to any other genes in the GenBank database, but they do correspond to several expressed sequence tags (*e.g.*, yj73f03.r1 and ys02h12.r1, respectively). They encode putative proteins that are 244 and 300 amino acids long, respectively (Fig. 3*A*), which can be translated *in vitro* into proteins that migrate with the expected mobilities (Fig. 3*B*). This suggests that these genes could generate functional proteins in S91 cells and in the analyzed tissues as well.

Clone 10d, on the other hand, is the highly conserved murine homologue of a human gene named BM28 (*CDCL1*; Ref. 24; 95% identity at the amino acid level, see Fig. 3*A*). This gene is a member of an extensive family of early S-phase proteins that also contains the *Saccharomyces cerevisiae* genes *CDC46*, *MCM2*, and *MCM3*, the *Schizosaccharomyces pombe* gene *CDC21*, and the murine P1 protein (36). BM28 has been reported to have two important functions: it is essential for both entry into S phase and cell division (25). Although the mode of action of these proteins is unclear, they contain a putative ATP-binding domain with the consensus sequence IDEFDKM (clone 10d/BM28 amino acids 586–592), which suggests that they function in an ATP-consuming step during the initiation phase of DNA replication.

BM28 is a phosphoprotein, which shuttles in and out of the nucleus in a cell cycle-specific fashion (37). Its human chromosomal location is 3q21, which is close to a chromosomal breakpoint in acute myeloid leukemia, and it was suggested that it may play a role in this disease (38). It is conceivable that this gene may give a growth advantage to a cell when it is overexpressed or deregulated and, as such, may act as an oncogene or growth promoter. This theory will be explored next.

Constitutive Expression of Clone 10d in S91 Cells Confers Dramatically Increased Resistance to RA. We reasoned that, if RA-dependent repression of clone 10d leads to growth arrest, than exogenously introduced clone 10d, by increasing the concentration of the gene product, should confer more resistance to RA. On the other hand, if this gene is not of critical importance, extra gene copies should make no functional difference. To test this hypothesis, S91 cells were permanently transfected with control vector pcDNA3 or with a vector which constitutively expresses clone 10d *via* the cytomegalovirus promoter (pcDNA10d). After selection in G418, two different control-transfected cell lines (lines 1 and 3) and two different pcDNA10d-transfected cell lines (lines 5 and 15) were obtained and propagated from individual neomycin-resistant colonies.

Next, we examined the sensitivity of these clonal cell lines to RA-induced growth arrest. Cells were treated for 5 days with increas-

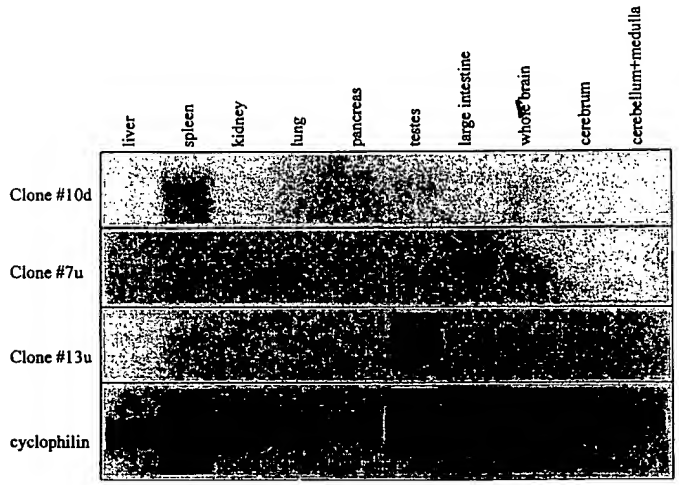

Fig. 2. Specific, differential expression of clones 7u, 13u and 10d in various normal mouse tissues. Total RNA was isolated and analyzed (about 15 μg/lane) by Northern blot. Cyclophilin expression serves as control.

A

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Clone #10d     PGQVADMAESSESLSASSPARQRRRISDPLTSSPGRSSRRADALTSSPGRDLPPFEDESEGLLGTE
                ||||||:. .|.: |||| .|||||||||||||.|||||||||||||||||||||||||||||| 60
BM28           EDRGTAMAESSESFTMASSPAQRRRGNDPLTSSPGRSSRRTDALTSSPGRDLPPFEDESEGLLGTE

GPMEEEEDGGELIGDGMERDYRPIPELDVYEAEGLALDDEDVEELTASQREAAERTMRQRDREAGRGLGRMRRGPLYDSS
||:|||||:|||||||||||||:|||||.|||||||||||||||||||||||||||||.|||||||||||||||| ||||. 140
GPLEEEEDGEELIGDGMERDYRAIPELDAYEAEGLALDDEDVEELTASQREAAERAMRQRDREAGRGLGRMRRGLLYDSD

EEDEERPARKRRHVERATEDGEEDEEMIESIENLEDLKGHSVREWVSMAGPRLEIHHRFKNFLRTHVDSHGHNVFKERIS
||||||||||||:|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 220
EEDEERPARKRRQVERATEDGEEDEEMIESIENLEDLKGHSVREWVSMAGPRLEIHHRFKNFLRTHVDSHGHNVFKERIS

DMCKENRESLVVNYEDLAAREHVLAYFLPEAPAELLQIFDEAALEVVLAMHPKYDRITNHIHVRISHLPLVEELRSLRQL
||||||||||||||||||||||||||||||||||||||||||||||.|||||||||||||||||||||||||||||| 300
DMCKENRESLVVNYEDLAAREHVLAYFLPEAPAELLQIFDEAALEVVLAMYPKYDRITNHIHVRISHLPLVEELRSLRQL

HLNQLIRTSGVVTSCTGVLPQLSMVKYNCSKCNFVLGPFCQSQNQEVKPGSCPECQSAGPFEINMEETIYQNYQRIRIQE
||||||||||||||||||||||||||.|||||||||||||||||||||||||||||:|||||||||||||||||||| 380
HLNQLIRTSGVVTSCTGVLPQLSMVKYNCNKCNFVLGPFCQSQNQEVKPGSCPECQSAGPFEVNMEETIYQNYQRIRIQE

SPGKVAAGRLPRSKDAILLADLVDSCKPGDEIELTGIYHNNYDGSLNTANGFPVPATIILANHVAKKDNKVAVGELTDED
||||||||||||:|||||||||||||||||||||||||||||||||||||||||||||||:|||||||||||||||||| 460
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VKMITGLSKDQQIGEKIFASIAPSIYGHEDIKRGLALALFGGEPKNPGGKHKVRGDINVLLCGDPGTAKSQFLKYIEKVS
|||||:|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 540
VKMITSLSKDQQIGEKIFASIAPSIYGHEDIKRGLALALFGGEPKNPGGKHKVRGDINVLLCGDPGTAKSQFLKYIEKVS

SRAIFTTGQGASAVGLTAYVQRHPVSREWTLEAGALVLADRGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVTSL
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 620
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HLRDYVIEDDVNMAIRVMLESFIDTQKFSVMRSMRKTFARYLSFRRDNNELLLFILKQLVAEQVTYQRNRFGAQQDTIEV

PEKDLMDKARQINIHNLSAFYDSDLFKFNKFSRDLKRKLILQQF*      Clone #10d
|||||:|||||||||||||||||:||::||||:|||||:||||| 904
PEKDLVDKARQINIHNLSAFYDSELFRMNKFSHDLKRKMILQQF*      BM28
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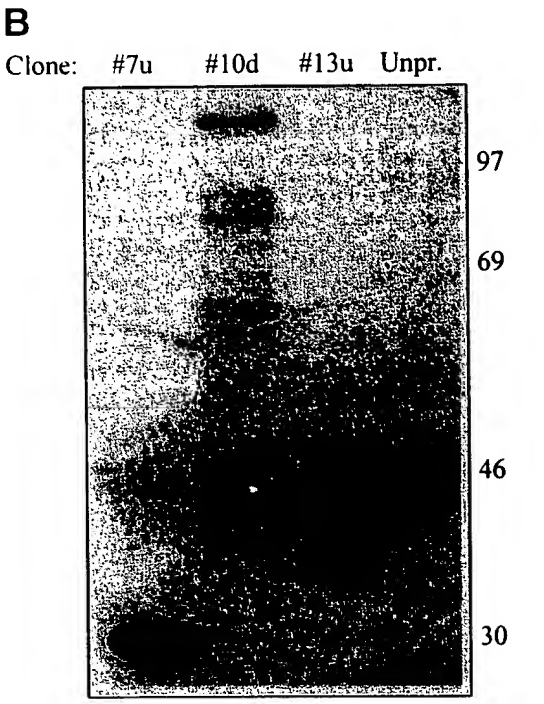


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Clone #7u    1  MGTPGEGLGR CSHALIRGVP ESLASGEGAG AGLPALDLAK AQREHGVLGG
            51  KLRQRLGLQL LELPPEESLP LGPLLGDTAV IQGDTALITR PWSPARRPEV
           101  DGVRKALQDL GLRIVEMGDE NATLDGTDVL FTGREFFVGL SKWTNHRGAE
           151  IVADTFRDFA VSTVPVSGSS HLRGLCGMGG PRTVVAGSSE AAQKAVRAMA
           201  ALTDHPYASL TLPDDAASDC LFLRPGLPGA TPFLLHRGGS AEAL*

Clone #13u   1  MLDSLLAIGG LVLLRDSVEW EGRSLLKALI KKSALRGEQV HVLGCEVSEE
            51  EFREGFDSDV NSRLVYHDLF RDPLNWSKPG EAVPEGPLKA LRSMCKRTDH
           101  GSVTIALDSL SWLLCHIPCV TLCQALHALS QQNGDPGDNS LVEQVHVLGL
           151  LHEELHGPGS MGALNTLAHT EVTLSGKVDQ TSASILCRRP QQRATYQTWW
           201  FSVLPDFSLT LHEGLPLRSE LHPDHHTTQV DPTAHLTFNL HLSKKEREAR
           251  DSLTLPFQFS SEKQKALLHP VPSRTTGRIF YEPDAFDDVD QEDPDDDLDI*
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Fig. 3. Protein sequence analysis (*A*) and *in vitro* translation (*B*) of the complete coding regions of cDNAs of differentially expressed genes in S91 cells that have been growth arrested by treatment with RA. Clone 10d is the homologue of the human BM28 (*CDCL1*) gene. Clones 7u and 13u are novel and have no homology to other known sequences. GenBank accession nos., AF004105, AF004106, and AF004107, respectively. [Note: Clone 10d was also cloned by H. Kimura (unpublished observations), GenBank accession no. D86725]. *A*, predicted amino acid sequences of clones 10d, 7u and 13u. Clone 10d (*top sequence*) and BM28 (*bottom sequence*) are aligned to illustrate the high evolutionary conservation between the two (862 of 904, or 95% identical residues). *Arrowhead*, first in-frame methionine. Ten amino acids downstream is another methionine in BM28 (indicated in *boldface*), which was thought to be the translational start site (24). However, this residue is not conserved in clone 10d, which makes it a less likely possibility. *B*, autoradiogram of 8% SDS-PAGE of [^{35}S]methionine-labeled, *in vitro* transcribed/translated cDNAs in the rabbit reticulocyte lysate system showing that the open reading frame in the cDNAs of clones 7u, 10d, and 13u can be translated into full-length proteins that migrate with their respective expected mobilities (*top band* in each lane). *Right*, protein size markers (in kDa). *Unpr.*, unprogrammed reticulocyte lysate.

ing doses of RA, as indicated in Fig. 4. Relative cell numbers were obtained by means of a colorimetric assay, in which A_{490} nM directly correlates with the number of viable cells. The absorbance values of control-treated cells were arbitrarily set at 100%, and all other values were normalized accordingly. The two control transfectants display a very similar dose-dependent block in proliferation, with an IC_{50} of about 5×10^{-8} M, as expected (39). However, the two pcDNA10d transfectants do not begin to show any RA-induced growth arrest until concentrations are between 10^{-8} M and 10^{-7} M; the IC_{50} of these lines is about 5×10^{-6} M, two logarithmic orders higher than the control transfectants. This result suggests that RA-induced growth arrest of S91 cells is largely mediated through repression of clone 10d.

Expression of Clone 10d/BM28 Correlates with Development of RA-resistant Human Melanoma. The above results indicate that clone 10d/BM28 may play an important role in the etiology of human melanoma. If expression of this gene is critical for tumor cell proliferation, as it is in S91 cells, and, by inference, is likely to promote tumor growth *in vivo*, then deregulated or otherwise aberrant expression of clone 10d/BM28 may confer a growth advantage to developing human melanoma as well. If this is the case, it can be expected that this gene is expressed in RA-resistant melanoma cells, but at much lower levels in benign melanocytes. In addition, melanoma cell lines derived from patient material are generally refractory to RA-induced growth arrest (our own observations; Fig. 5). This implies that the

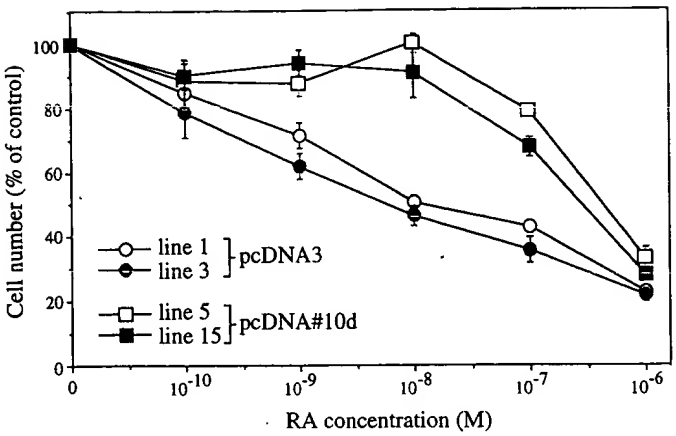

Fig. 4. Constitutive expression of clone 10d in S91 cells provides resistance against RA-induced growth arrest. Two different lines of control-transfected cells (○ and ●, lines 1 and 3 with plasmid pcDNA3, respectively) and two different lines of clone 10d-transfected cells (□ and ■, lines 5 and 15 with plasmid pcDNA10d, respectively) were cultured for 5 days in 96-well plates in the presence of the indicated amounts of RA. Cell numbers were determined by colorimetric assay, based on the cellular conversion of a tetrazolium salt into a formazan that is measured at 490 nm and that directly correlates with the number of viable cells. Absorbances of control-treated cells were arbitrarily set at 100%, and all other values were normalized accordingly. *Data points*, means of a representative experiment done in triplicate, which was repeated at least three times with similar results; *bars*, SD.

expression of this gene should not or should no longer be regulated by RA.

To investigate this theory, first, expression of clone 10d/BM28 was determined in 11 different melanoma cell lines, which, for the most part, were established in our laboratory (one of these, line C3B, is derived from a recurring tumor from a patient from which we previously derived line C3A; see Fig. 5*A*) and compared to the levels in a pure culture of primary human melanocytes. RT-PCR analysis was used to measure expression levels of clones 10d and BM28, and expression of β-actin was used as internal control. Results are shown in Fig. 5*A*. To ascertain that the results from the RT-PCR analysis are in accordance with the previously obtained data by Northern blot analysis, we included S91 cells that were treated for 2 days with DMSO (control) or 1 μM RA. As shown in *Lanes 1* and *2*, respectively, clone 10d is clearly expressed in the untreated cells but repressed in the RA-treated cells. Thus, the results from these two different types of detection methods are in good agreement with one another. Clone 10d can also be easily detected by RT-PCR in (untreated) B16 murine melanoma cells (*Lane 3*), which is also in accordance with Northern blots (data not shown). We then analyzed 11 (untreated), RA-resistant human melanoma cell lines (*Lanes 4–14*) and the primary melanocytes (*Lane 15*) for expression of BM28. As shown, BM28 gene expression levels appear somewhat variable as compared to the evenly intense β-actin controls, but expression is clearly detectable in *Lanes 4–10, 12,* and *14*, which constitutes 80% (9 of 11) of cell lines. In contrast, expression is virtually undetectable in the (dividing) primary melanocytes (*Lane 15*). RA treatment has no effect on expression in these cells (*Lane 16*) or in any of the human melanoma cells (data not shown). These results are consistent with our postulated hypothesis.

A second condition that should be met to establish a putative role for BM28 in human melanoma is that BM28 expression should not be down-regulated by RA in BM28-positive cells, because these cell lines are RA resistant. Our previous methods are all mRNA based, and for this experiment, we chose a technique to monitor protein expression levels of clone 10d/BM28. Fig. 5*B* shows a Western blot that was loaded with equal amounts of whole-cell extracts of S91 cells (confirmed by Coomassie Brilliant Blue staining of the gel and probing with lamin A antibody; data not shown), which were treated for 5 days with DMSO (control; −) or with 1 μM RA (+). The blot was probed with an antihuman BM28 antibody, which cross-reacts with clone 10d due to its extremely high sequence conservation (Fig. 3*A*). As shown in Fig. 5*B*, expression of clone 10d is prominent in untreated cells, and as expected, RA treatment leads to repression of expression at the protein level as well. Thus, these data are in complete accordance with our previous results. We then did the same type of experiment with two human melanoma cell lines that express high levels of BM28, lines A375 and C6 (corresponding to *Lanes 14* and *10* in Fig. 5*A*, respectively). As can be seen from Fig. 5*B*, BM28 protein levels are not affected by up to 5 days of RA treatment. We also confirmed this by RT-PCR (data not shown).

These results are consistent with and provide evidence for the hypothesis that aberrant expression of clone 10d/BM28 may play an important role in the etiology of human melanoma by conferring a growth advantage to the neoplastic cell.

Evidence That Clone 10d, in Contrast to Clone 7u, Is a New Target Gene for RARs. Finally, we wanted to answer the original question of whether clone 10d represents a new RAR target gene, or whether it is a secondary response gene. This is an important issue because it provides information about the order of the flow of information after the addition of RA.

First, we determined the kinetics of RA-dependent regulation of gene expression by performing a time course analysis. RA (1 μM) was added for 0 (control), 2, 8, 24, and 48 h to the cells, poly(A)$^+$ RNA was isolated, and gene expression was analyzed by Northern blot. In addition to our analysis of clone 10d, we included clone 7u because this gene is also extensively regulated. The results are shown in Fig. 6*A*. Interestingly, the two clones display reciprocal changes in gene expression that become clearly detectable between 8 and 24 h of treatment. Regulation appears to be maximal after 48 h in both cases. This time frame could be indicative of an early response-type of regulation of these two genes.

In our next experiment, we approached this question more directly. Cells were untreated or treated with 1 μM RA for 24 h, in the absence or presence of two different, sublethal concentrations (0.1 and 0.5

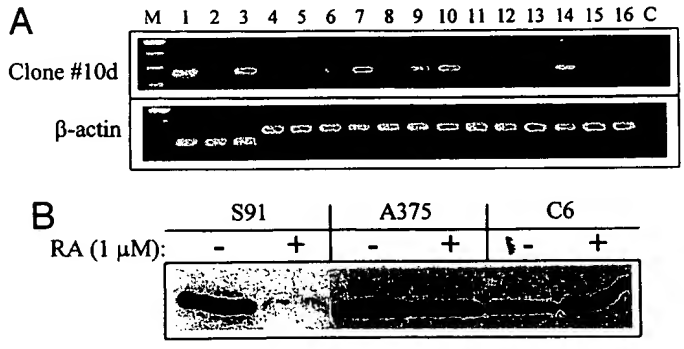

Fig. 5. Expression of clone 10d/BM28 in murine and human melanoma cell lines and primary human melanocytes. *A*, RT-PCR analysis of expression of clone 10d/BM28 by 1.5% agarose gel electrophoresis. β-Actin serves as control. Cells were grown in the absence of RA, except in *Lanes 2* (S91 cells) and *16* (human primary melanocytes), in which 1 μM RA was added to the medium for 48 h. Expected PCR product sizes are 363 bp for clone 10d/BM28 and 348 bp and 406 for murine and human β-actin, respectively. *Lane M*, 100-bp DNA ladder (*top band*, 600 bp); *Lane C*, control PCR without template. Murine melanoma cell lines: *Lane 1*, S91; *Lane 2*, S91 plus RA; *Lane 3*, B16. Human melanoma cell lines: *Lane 4*, C1; *Lane 5*, C2; *Lanes 6* and *Lane 7*, C3A (primary tumor) and C3B (recurring tumor), established from same patient; *Lane 8*, C4; *Lane 9*, C5; *Lane 10*, C6; *Lane 11*, C8; *Lane 12*, C9; *Lane 13*, C12; *Lane 14*, A375. Human primary melanocytes: *Lanes 15* and *16*, without and with RA, respectively. *B*, S91 cells and human melanoma lines A375 and C6 were grown for 5 days in the absence (−) or presence (+) of 1 μM RA. Cells were harvested and total cellular proteins were extracted, separated by 8% SDS-PAGE, and immunoblotted with polyclonal anti-BM28 antibodies that cross-react with clone 10d (see "Materials and Methods").

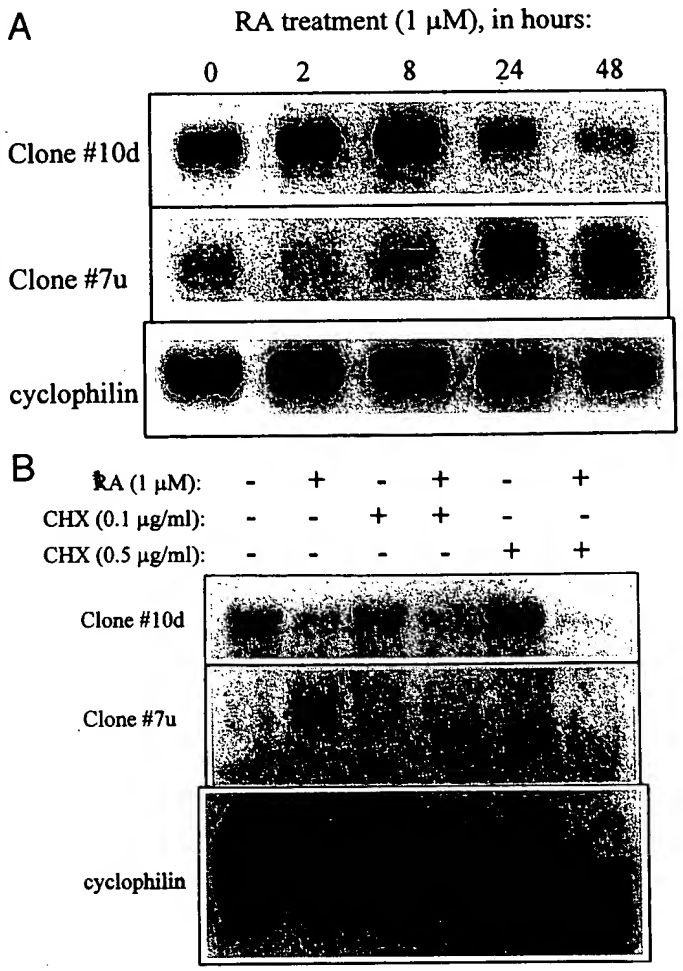

Fig. 6. Time course (*A*) and mechanism (*B*) of RA-dependent regulation of expression of clones 10d and 7u in S91 cells. *A*, Northern blot analysis (1.5 μg of double-selected poly(A)$^+$ RNA/lane), showing that the expression of clones 10d and 7u changes significantly in a reciprocal fashion between 8 and 24 h of treatment. Regulation is maximal after 48 h. Cyclophilin expression serves as control. *B*, Northern blot analysis (1 μg of poly(A)$^+$ RNA/lane), showing that induction of expression of clone 7u is abrogated in a dose-dependent fashion by the protein synthesis blocker CHX, whereas repression of expression of clone 10d is unaffected. This suggests that clone 10d, in contrast to clone 7u, may be a direct target gene for RARs. Cyclophilin expression serves as control.

μg/ml, respectively) of the protein synthesis blocker CHX. These amounts are sufficient to reduce protein synthesis levels to about 10% and less than 1% of those of control-treated cells, respectively (data not shown). Forty-eight-h treatment could not be performed here because the cells cannot withstand treatment with CHX over this period. However, as shown above, significant regulation takes place between 8 and 24 h of treatment. Poly(A)$^+$ RNA was then extracted, and expression of clone 10d and 7u was determined by Northern blot analysis (Fig. 6*B*). Cyclophilin expression serves as the internal control. Untreated S91 cells contain functional RARs, and thus, if these genes are indeed RAR-target genes, it can be expected that CHX will not affect their RA-dependent mode of regulation. On the other hand, if they are not primary target genes and require induction of other regulatory factors, RA-dependent regulation may be abrogated. Fig. 6*B* shows that, as expected, RA again represses clone 10d expression, and induces clone 7u. Addition of CHX does not affect RA-dependent repression of clone 10d. In contrast, induction of clone 7u is blocked by CHX in a dose-dependent way. These results suggest that clone 10d is a novel, negatively regulated RAR-target gene, whereas clone 7u most likely represents a secondary response gene that is not directly induced by RARs.

DISCUSSION

S91 melanoma cells provide an excellent model system for the neoplastic change of melanocytic cells into melanoma. This process is completely retinoid dependent and can, therefore, be controlled. Here, we investigated the process of RA-induced growth arrest, which precedes morphological differentiation. Our rationale is that the genes involved in these processes may play a role in the development of melanoma. By using subtractive hybridization and subsequent cloning techniques, we succeeded in obtaining four cDNAs with complete open reading frames, which are differentially expressed during RA-induced growth arrest of S91 melanoma cells. One of these is cyclin D1, which was previously shown to be repressed in terminally differentiated melanoma cells. Our results show that this also happens during reversible differentiation. Two of our clones represent novel genes with no apparent homology to other sequences in the GenBank database. However, their RA-dependent induction and association with growth arrest in these cells, and their surprisingly different tissue-specific expression pattern indicates that they may serve a specific physiological function, but this question cannot yet be answered.

Fortunately, we were able to at least partly address this issue with clone 10d, which is repressed after RA treatment at both the mRNA and protein levels. It is the homologue of the human gene BM28 (*CDCL1*), a gene that is essential for mitosis and cell division (25), and in S91 cells, its expression is associated with tumor cell proliferation. Transfection studies show that RA-induced growth arrest is, to a large extent, mediated through repression of this gene, although other genes are likely also involved (40). This may explain why, at very high concentrations of RA (10^{-6} M), the pcDNA10d transfectants are also blocked in their proliferation: other factors like cyclin D1 could become rate limiting, or tumor growth suppressors could be induced. It could also simply be that at those concentrations, the amount of clone 10d may fall below critical threshold levels that are necessary for continued cell division. Thus, high expression levels of clone 10d ensure cellular proliferation, and it is conceivable that this gene may promote tumor growth, particularly in melanoma. Our evidence suggests that this is, indeed, the case. Clone 10d/BM28 is clearly expressed in 80% of human malignant melanoma cell lines, but it is virtually undetectable in benign primary melanocytes (Fig. 5*A*). Human melanoma cells are generally resistant to RA, and consistent with its putative role as an oncogene in this disease, we find that clone 10d/BM28 is not regulated by RA in these cells. Our evidence suggests that clone 10d is directly repressed by RARs in S91 cells, although indirect regulatory mechanisms *via* liganded-RAR-mediated anti-AP1 activity, cannot completely be ruled out. It is not known why this does not occur in human cells; it may be that one or more defects in the RA response pathway have already accumulated when human tumors present themselves clinically, or there could be an intrinsic difference in the regulation of this gene between mouse and man. However, given the very high sequence conservation of clone 10d and BM28 and the potential functional consequences of deregulated expression, it is quite likely that normally other control mechanisms are in place that could have broken down during the development of human disease. This question can be answered when the promoters of both clone 10d and BM28 are cloned and analyzed for potential RA response elements and/or other regulatory elements.

The incidence of melanoma has been rising steadily, and an estimated 1 in 80 Americans will develop this disease (41, 42). There is no effective cure for advanced disease, and there is a great need for animal models to develop and test new therapies. Recently, a transgenic mouse model was created by Bradl *et al.* (43), who targeted expression of the SV40 large T antigen to melanocytes. Although this

model has provided very useful information, it may not accurately reflect the etiology and genetics of human disease, which arises from complex events apparently involving many different genes (44–46). The tumors which derive from these mice are also extremely aggressive, which makes them difficult to use for novel therapeutical treatments because the animals rapidly succumb. By cloning differentially expressed genes from the S91 cell model and characterizing the function of these genes *in vitro*, we hope to obtain putative tumor suppressors and tumor promoters that may play a role in this disease. Ultimately, we may have to assess the *in vivo* function of clone 10d/BM28 and yet-to-be-derived genes from the S91 model in melanoma and other cancers, by creating transgenic mouse strains that aberrantly express these genes. This could provide alternative genetic animal models for this disease that may eventually lead to the design of more effective treatments and/or diagnostics.

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